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ARTICLE PRINCIPAL

Recouvrant en partie

LA THESE DE DOCTORAT ES SCIENCES Naturelles

présentée

A LA FACULTE DES SCIENCES

de PARIS

par

DENISE PAULIN

pour obtenir

le grade de Docteur Es-Sciences

Sujet de la Thèse : PEPTIDYL-tARN HYDROLASE d'ESCHERICHIA COLI

soutenue le 18 Décembre 1970

devant la Commission d'Examen

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Enzymatic Hydrolysis of N-Acyl-Aminoacyl Transfer RNAs

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In a rapidly growing culture of *E. coli*, each cell contains approximately 30,000 ribosomes. For each ribosome, there are about 10 tRNA molecules out of the 40 or more existing species. Thus it is likely that during intense protein synthesis, at least certain tRNA molecules constitute a limiting factor in the rate of polypeptide chain growth. Any mechanism that renders aminoacyl-tRNA molecules unavailable for translation may be detrimental for the cell, since it would further decrease the number of functional tRNA molecules. Such would be the case, for instance, if growing peptide chains were accidentally released with their tRNAs from the protein-synthesizing machinery. In the cell, tRNA would not be recovered from peptidyl-tRNA, because the ester bond which unites the two parts of the molecule is very stable and its spontaneous hydrolysis is improbable. Furthermore, aminoacyl-tRNA synthetases are certainly unable to regenerate tRNAs from peptidyl-tRNAs, even if AMP and pyrophosphate were available, for they do not recognize N-blocked aminoacyl-tRNAs. For the formation of an *E. coli* cell, 1×10^6 protein molecules of average mol wt = 40,000 must be synthesized and therefore approximately 4×10^8 codon-translations are required. If the probability of peptidyl-tRNA release at the translation of each codon were one per thousand, the total supply of tRNAs within the cell would be exhausted by the time only half of the codon-translations had occurred. In vivo, it is as yet very difficult to assess whether such "accidents" can occur, and if so, at what rate. It is not unlikely that certain codon-anticodon interactions are much weaker than others; this could favor the release of peptidyl-tRNAs prior to the completion of the polypeptide chain and would lead even more rapidly to a halt in protein synthesis, by subtracting certain species from the tRNA pool. The breakdown of polysomes observed during amino acid starvation (Morris and DeMoss, 1966), may also be responsible for the formation of free peptidyl-tRNAs. A thorough examination of the data obtained in vitro and reported by several investigators (Bretscher et al., 1965; Takanami and Yan, 1965; Ganoza and Nakamoto, 1966) shows that with synthetic messengers carrying no terminating codon, as many as 10 to 20% of the peptides formed can be found

dissociated from the ribosome-messenger complex either as free peptides or as peptidyl-tRNAs. The spontaneous release of short peptides bound or unbound to tRNAs was also observed with natural messengers such as R17 or f2 RNAs (Capecci, 1967; Bretscher, 1968).

The existence in vivo of free peptidyl-tRNAs may have another dramatic consequence for the cell. It could possibly lead to chain initiation by these peptidyl-tRNAs at other codons along the messenger than at the initiation codon. Such unphysiological initiations seem to be a possibility, as suggested by in vitro experiments with acetyl-aminoacyl-tRNAs (Haenni and Chapeville, 1966; Verhoef et al., 1967; Laycock and Hunt, 1969) or oligopeptidyl-tRNAs (Rychlick, 1966; Gottesman, 1967).

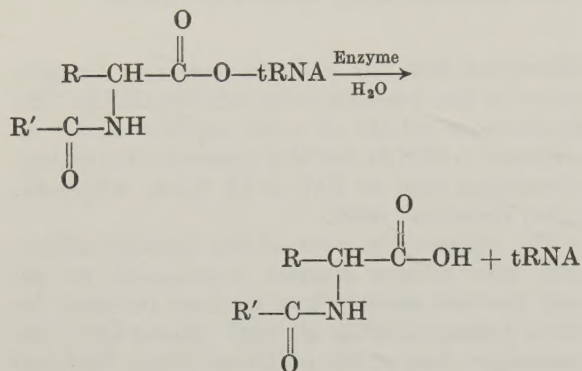
Consequently, it is likely that there exists within the cell a mechanism whereby tRNAs can be regenerated from peptidyl-tRNAs. An enzyme, the acyl-aminoacyl-tRNA hydrolase, that catalyzes such a reaction has been isolated in our laboratory (Cuzin et al., 1967). A factor presenting identical properties has since been described by several investigators (de Groot et al., 1968; Kössel and RajBhandary, 1968; Vogel et al., 1968b). It was at first thought that this hydrolase might in some manner cooperate in the process of polypeptide chain termination. However, as a result of various observations by Capecci (1967), and Scolnick et al. (1968), it appears unlikely that this hydrolase participates in chain termination. On the other hand, the hypotheses put forward in the preceding paragraphs, together with the experimental results to be presented here, strongly suggest that the physiological role of this enzyme is to liberate tRNAs from peptidyl-tRNAs which have escaped the ribosome-messenger complex, or from free acyl-aminoacyl-tRNAs in which the amino acid moiety has been accidentally acylated.

HYDROLYSIS OF ACYL-AMINOACYL-tRNAs

The hydrolase used throughout the experiments to be described was obtained from extracts of *E. coli* MRE 600, and was purified 500-fold from the S-150 supernatant fraction. It is a positively charged protein of a mol wt of around 20,000. It is

not retained by DEAE-cellulose at pH 7.4, and in this respect behaves as does the initiation factor F_1 (Salas et al., 1967); however, it differs from F_1 in its sensitivity to heat: the hydrolase is rapidly inactivated by heating at 60°C whereas F_1 is not (Vogel et al., 1968b).

The hydrolase splits the ester bond between the amino acid and the tRNA of aminoacyl-tRNAs with a blocked α -amino group on the amino acid.



When aminoacyl-tRNAs acetylated chemically (Haenni and Chapeville, 1966) were used as substrates, free acetyl-amino acids and tRNAs were formed (Fig. 1). It has also been shown that peptidyl-tRNAs bearing either a free or a blocked α -amino group on the amino acid are hydrolyzed, such as diPhe-tRNA, acdiPhe-tRNA, acLeu-Gly-Phe-tRNA, and several others. Figure 2 shows the effect of the hydrolase on acLeu-Gly-Phe-tRNA. Of the numerous peptidyl-tRNAs tested, only polyLys-tRNA is not hydrolyzed; however, this compound must possess an unusual configuration which could account for its nonrecognition by the enzyme. No metal ions other than those strongly bound to tRNA and not removed by dialysis are required for enzymic hydrolysis. High salt concentrations inhibit the reaction. The hydrolase is insensitive to the presence or absence of thiols; it is not inhibited by parachloromercuribenzoate. It has a pH optimum above 8 but hydrolysis is already rapid at pH 7. Uncharged tRNA at a concentration 50 or 100 times greater than that of the substrate behaves as a competitive inhibitor of the reaction (Fig. 3). Other polyanions, such as ribosomal RNA, synthetic polynucleotides, DNA, and heparin also inhibit the reaction.

During the process of purification, it was observed that although most of the enzyme remained in the soluble fraction, some hydrolase activity was strongly bound to ribosomes, and was released only by several washings of the ribosomes in high salt (0.1 M NH_4^+ and 0.01 M Mg^{++}). Salt-washed ribosomes were treated with urea and LiCl which do not inactivate the hydrolase, and

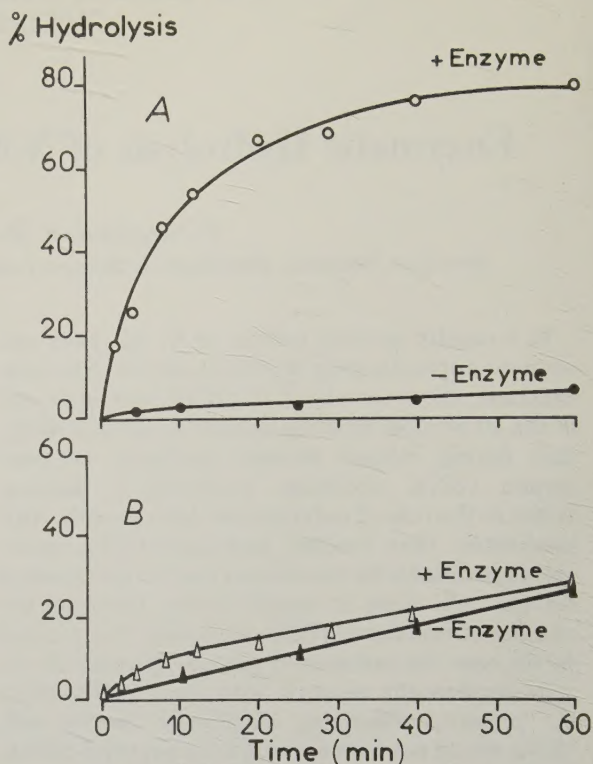


FIGURE 1. Enzymatic hydrolysis of acVal-tRNA. Mixtures of 0.2 ml containing: 20 μ moles of sodium barbital-acetic acid pH 8.6; 0.8 $m\mu$ moles of ac-¹⁴C-Val-tRNA; 0.8 μ moles of ³H-Val-tRNA and, when indicated, 40 μ g of hydrolase were incubated at 37°C. At various time intervals, aliquots were removed and analyzed by paper electrophoresis in 0.5 M formic acid at 20 v/cm for 90 min. The spots corresponding to unhydrolyzed material, to ac-¹⁴C-Val, and to ³H-Val were cut into strips, and the radioactivity was determined. A: % hydrolysis of ac-¹⁴C-Val-tRNA. B: % hydrolysis of ³H-Val-tRNA.

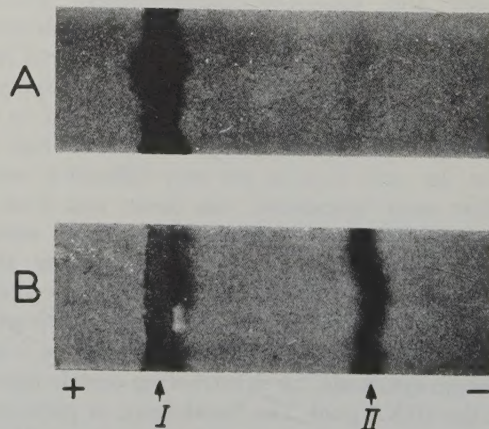


FIGURE 2. Enzymatic hydrolysis of acLeu-Gly-¹⁴C-Phe-tRNA. Mixtures containing in 25 μ l: 1 μ mole of sodium barbital-acetic acid pH 8; 10 μ moles of the peptidyl-tRNA (prepared according to Lapidot et al., 1967); and 2 μ g of hydrolase in A but not in B, were incubated for 5 min at 37°C. The samples were electrophorized as indicated in Fig. 1. The autoradiogram shows in I: unhydrolyzed material, and in II: free acetylpeptide.

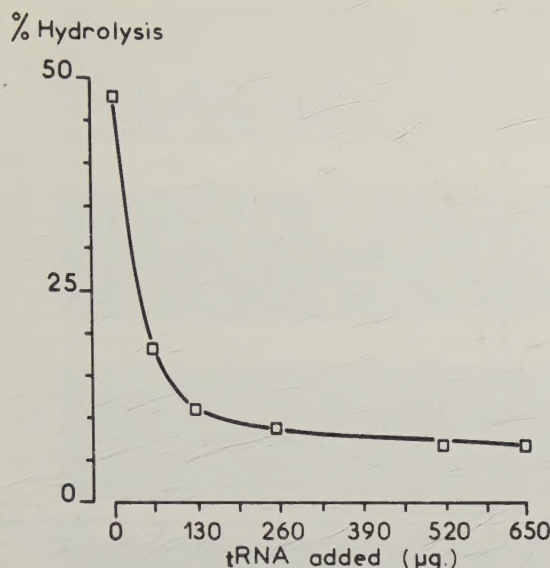


FIGURE 3. Inhibition of acLeu-tRNA hydrolysis by uncharged tRNA. To 50 μ moles of partially purified ac- 14 C-Leu-tRNA (tRNA^{Leu} was 38% pure) and 1 μ mole of sodium-barbital-acetic acid pH 8 in 60 μ l were added varying amounts of crude uncharged tRNA. After 5 min at 37°C, the samples were analyzed as described in Fig. 1.

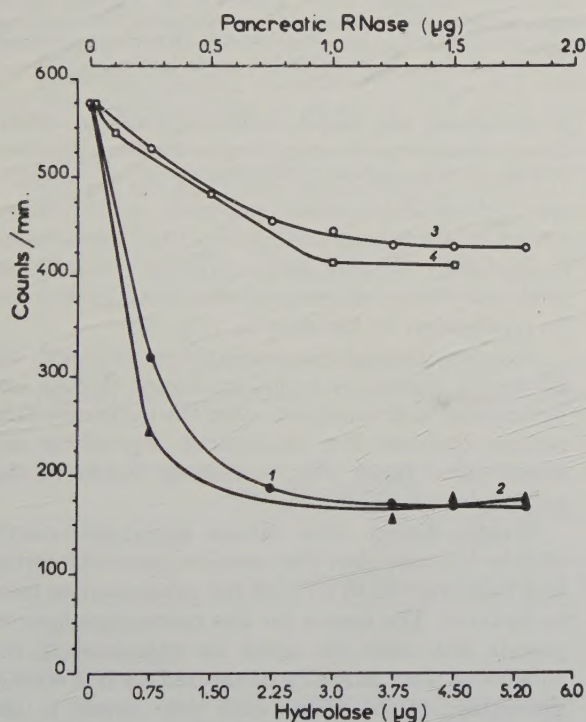


FIGURE 4. Effect of hydrolase and of pancreatic RNase on acPhe-tRNA bound to ribosome-poly U. Each incubation mixture contained in a final volume of 50 μ l: 2.5 μ moles of Tris-HCl pH 7.2; 1 μ mole of magnesium chloride; 5 μ moles of NH_4Cl ; 2.5 μ moles of KCl; 3.3 μ moles of ac- 14 C-Phe-tRNA; and, where indicated, 2.5 μ moles of base residues of poly U; 2.3 A_{260} units of *E. coli* ribosomes. After a preincubation period of 10 min at 24°C, varying amounts of

the ribosomal proteins were extracted. These proteins showed no hydrolase activity, thereby suggesting that the hydrolase is not a ribosomal constituent. When salt-washed 50 S and 30 S ribosomal particles were incubated with hydrolase, the amount of enzyme bound was proportional to the content in nucleic acid of each subunit.

Since the hydrolase adsorbed onto ribosomes remains capable of hydrolyzing free acetyl-aminoacyl-tRNAs, it appeared of interest to investigate the behavior of this enzyme toward a ribosome-bound substrate. The Millipore binding assay of Nirenberg and Leder (1964) was used, and acPhe-tRNA bound in the presence of poly U served as substrate. Figure 4 shows that when acPhe-tRNA is bound to the ribosome-messenger complex, it is not hydrolyzed; it is protected from the action of the hydrolase to the same extent that it is protected from the action of pancreatic RNase (Pestka, 1968). Similarly, peptidyl-tRNAs tested under identical conditions are protected from the hydrolase (de Groot et al., 1968). It appears from these results that the hydrolase, free or in contact with ribosomes, acts only with free peptidyl-tRNAs not engaged in a complex with the ribosomal intermediate. Therefore, the enzyme does not hinder the normal process of peptide chain elongation.

SPECIFICITY OF THE HYDROLASE

THE ACYL-AMINOACYL-MOIETY

The nature of the amino acid does not greatly influence the rate of hydrolysis, as indicated by results obtained with several acyl-aminoacyl-tRNAs (Cuzin et al., 1967). This also implies that the primary structure of the tRNA moiety does not significantly modify the rate of hydrolysis.

On the other hand, the substituent on the amino acid is important: acetylated compounds are apparently hydrolyzed faster than the corresponding formylated derivatives, as indicated in Fig. 5 (Paulin et al., 1968). Kössel and RajBhandary (1968) and Vogel et al. (1968b) have examined the fate of carbobenzyloxylated compounds, and have shown them to be hydrolyzed even more rapidly than are the acetylated derivatives. De Groot has observed that the hydrolysis of oligoGly-tRNAs increases with increasing length of the peptide up

hydrolase or of pancreatic RNase (Pestka, 1968) were added, and incubation continued for an additional 15 min at 24°C. The unhydrolyzed acPhe-tRNA was precipitated by cold TCA and counted. 1. +hydrolase + poly U; 2. +hydrolase + ribosomes; 3. +hydrolase + poly U + ribosomes; 4. +RNase + poly U + ribosomes. In a separate experiment, it was determined by Millipore filtration that after the preincubation period, all the acPhe-tRNA added was bound to the ribosome-poly U complex.

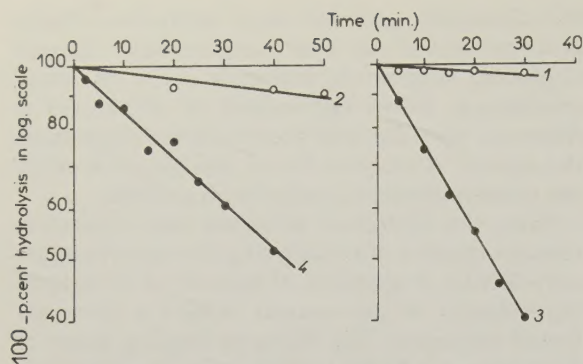


FIGURE 5. Enzymatic hydrolysis of fVal-tRNA (curve 4) and acVal-tRNA (curve 3). Incubation mixtures containing 10 μ moles of sodium barbital-acetic acid pH 8; 150 μ moles of f- 14 C-Val-, or ac- 14 C-Val-tRNA and, as indicated, 15 μ g of hydrolase were incubated in a final volume of 150 μ l at 37°C. At different times, aliquots were removed, and the 14 C-Val derivatives were separated, as in Fig. 1, by paper electrophoresis. 1 and 3: acVal-tRNA without or with enzyme. 2 and 4: fVal-tRNA without or with enzyme.

to pentaGly-tRNA and then remains constant (pers. commun.). Thus a correlation seems to exist between the size or hydrophobic nature of the acyl group and the rate of enzymatic hydrolysis. An amide group is probably a prerequisite for hydrolysis, since the hydrolase does not recognize α -hydroxy derivatives (Table 1).

THE tRNA MOIETY

The *E. coli* hydrolase interacts equally well with homologous (*E. coli*) or with heterologous (yeast, plant, animal) acetyl-aminoacyl-tRNAs. When acetyl-aminoacyl-tRNAs have been extensively treated with NO_2H , so as to deaminate an average

TABLE 1. ABSENCE OF HYDROLYSIS OF CHEMICALLY MODIFIED SUBSTRATES

Substrates	% Hydrolysis
α -hydroxy-isovaleryl-tRNA ^{Val} (I)	5
acVal-tRNA ^{Val}	90
α -hydroxy-caproyl-tRNA ^{Leu} (II)	8
acLeu-tRNA ^{Leu}	85
acVal-tRNA ^{Val} , partly deaminated (III)	4

Derivatives I and II were obtained by mild treatment of the corresponding 14 C-aminoacyl-tRNAs with 2 hr incubation at 0°C in a half-saturated solution of NaNO_2 containing 1% acetic acid. The pH of the mixture was 6 (Hervé and Chapeville, 1965). The capacity of derivatives I and II to bind to the ribosome-polynucleotide complex was unchanged as compared to the corresponding aminoacyl-tRNAs; after stripping, their recharging capacity was about 70%. Derivative III was obtained from acVal-tRNA^{Val} by a more drastic treatment for 8 hr at 20°C in 3 M NaNO_2 at pH 3.2 (Carbon, 1965). In this case, about 4 or 5 bases per tRNA molecule were deaminated. This deaminated tRNA could not be recharged, but still maintained its binding capacity (Hervé, 1966). The substrates (150 μ moles) were incubated in 150 μ l with 10 μ moles of sodium barbital-acetic acid pH 8.6 and 15 μ g of hydrolase for 30 min at 37°C. The incubation mixtures were then analyzed as in Fig. 1.

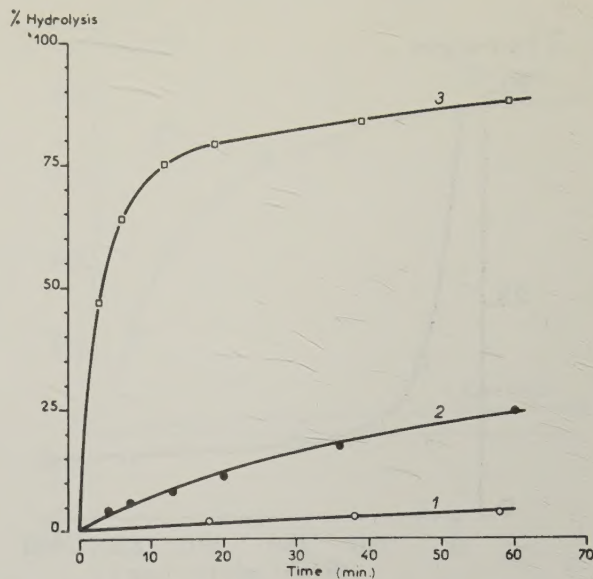


FIGURE 6. Enzymatic hydrolysis of EDTA-treated acVal-tRNA. Ac- 14 C-Val-tRNA was dialyzed overnight against 0.01 M EDTA in 0.01 M cacodylate, pH 6.5, then extensively against H_2O , and hydrolysis was examined. The incubation mixtures contained in 160 μ l: 5 μ moles of sodium barbital-acetic acid pH 8.6; 750 μ moles of ac- 14 C-Val-tRNA and, where indicated, 25 μ g of hydrolase, and were incubated at 37°C. Aliquots were removed at various times and analyzed. 1. untreated ac- 14 C-Val-tRNA without enzyme; 2. EDTA-treated ac- 14 C-Val-tRNA + hydrolase; 3. untreated ac- 14 C-Val-tRNA + hydrolase.

of five bases per tRNA molecule (Carbon, 1965), they no longer serve as substrates for the hydrolase (Table 1). The rate of hydrolysis of acVal-, acLeu-, and acIle-tRNAs whose secondary and tertiary structures have been modified by EDTA treatment is drastically reduced, emphasizing the importance of the secondary structure of the tRNA moiety for its recognition by the enzyme (Fig. 6).

Acetyl-aminoacyl-oligonucleotides obtained by RNase T₁ digests of acetyl-aminoacyl-tRNAs are hydrolyzed to some extent when the polynucleotide residue contains five nucleotides; hydrolysis becomes more rapid with increasing length of the polynucleotide chain (Fig. 7).

Kinetic studies with various acetyl-aminoacyl-tRNAs indicate that the reaction generally comes to a halt after 85 to 90% of the substrate has been hydrolyzed. The reason for this incomplete hydrolysis is not clear. It could be explained if the substrates were partially denatured, or if a certain percentage of the amino acid were bound to the tRNA in the 2' rather than in the 3' position and were not to equilibrate in the experimental conditions used.

ACYL-MET-tRNA^{Met}

The acylated derivatives of Met-tRNA^{Met} present unique characteristics and the hydrolysis

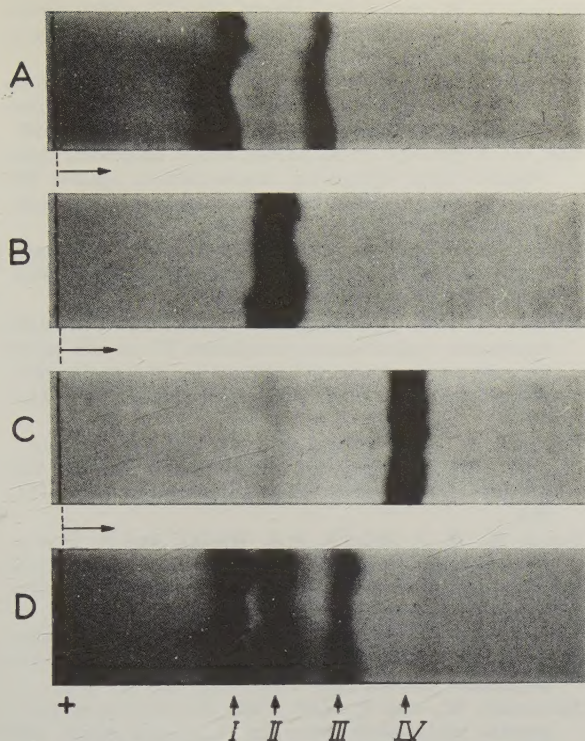


FIGURE 7. Enzymatic hydrolysis of acVal-oligonucleotides. Mixtures of 60 μ l containing 20 μ moles of ac- 14 C-Val-ACCAC (I) (Yaniv, pers. commun.), and 60 μ moles of ac- 14 C-Val-ACCACCCACUACU (III) (Yaniv and Barrell, 1969), were incubated for 15 min at 37°C with 3 μ moles of sodium barbital-acetic acid pH 8.6 and 10 μ g of hydrolase as indicated. The samples were electrophorized as in Fig. 1. The corresponding autoradiograms are shown here. A. no hydrolase; B. no hydrolase, but stripping by 0.1 N NaOH after incubation; C. no hydrolase, but in the presence of 10 μ g of pancreatic RNase (IV = ac- 14 C-Val-adenosine); D. with hydrolase (II = ac- 14 C-Val). When the radioactivity of the spots was determined it was found that the hydrolysis of (I) was 30% and that of (III) was 15%.

of these compounds has led to contradictory results (Kössel and RajBhandary, 1968; Vogel et al., 1968a;

TABLE 2. INFLUENCE OF pH ON THE HYDROLYSIS OF ACYL-MET-tRNA_f^{Met}

Substrates	% Hydrolysis	
	Tris pH 7.2	Barbital pH 8
fMet-tRNA _f ^{Met}	2	26
acMet-tRNA _f ^{Met}	5	27
acVal-tRNA _f ^{Val}	29	77

Mixtures of 60 μ l containing: 200 μ moles of pure f- 14 C-Met-tRNA_f^{Met} or ac- 14 C-Met-tRNA_f^{Met}, or 100 μ moles of unpurified ac- 14 C-Val-tRNA_f^{Val} were incubated for 15 min at 37°C with 8 μ g of hydrolase and 2 μ moles of sodium barbital acetic acid pH 8, or 5 μ moles of Tris-HCl pH 7.2; following incubation they were analyzed as in Fig. 1. From the values obtained were subtracted the amounts of nonenzymatic hydrolysis determined separately at each pH.

Paulin et al., 1968). We have shown that at pH values equal to, or higher than 8, fMet-tRNA_f^{Met} is rapidly hydrolyzed by the enzyme, whereas other investigators observed no hydrolysis at or near pH 7. Such discrepancies might reflect differences in the enzymatic preparations used; however, this is not the case: when the hydrolysis of acetyl-aminoacyl-tRNAs and of acMet- or fMet-tRNA_f^{Met} are compared, it is clear that the derivatives of Met-tRNA_f^{Met} are hydrolyzed at pH 8 but not at pH 7 (Table 2). The peculiar behavior of the Met-tRNA_f^{Met} derivatives probably corresponds to modifications of the tRNA_f^{Met} molecule, rendering it inaccessible to enzyme action at pH 7. With other acyl-aminoacyl-tRNAs like acyl-Met-tRNA_m^{Met} (Vogel et al., 1968a), such modifications may not occur, or may be without effect.

CONCLUSIONS

Results describing enzymatic hydrolysis of oligopeptidyl- and acetyl-aminoacyl-tRNAs suggest a possible physiological role for the enzyme, as has been discussed. This enzyme may be involved in the regeneration of tRNAs from oligopeptidyl-tRNAs which may be released from the protein synthesizing system prior to chain termination. It may also regenerate tRNAs from acyl-aminoacyl-tRNAs which might be formed by fortuitous acylation of amino-acyl-tRNAs by the acylating compounds abundant within the cell, such as the acylated derivatives of coenzyme A. In vivo experiments have recently shown that Leu-tRNA derivatives are formed that resist alkaline hydrolysis (Yegian and Stent, 1969). It is tempting to postulate that such compounds correspond to the acyl-aminoacyl-tRNAs formed by the mechanism just described.

The F-met-tRNA_f^{Met} derivative, which is not hydrolyzed at a pH close to 7 (probably the physiological pH of the cell), may not be disturbed as polypeptide chain initiator. The nonrecognition of this tRNA by the hydrolase, although surprising, is not unique: for example, the formylating enzyme distinguishes between Met-tRNA_f^{Met} and Met-tRNA_m^{Met}; likewise, in the presence of GTP, the elongation factor T does not interact with Met-tRNA_f^{Met} although it does interact with Met-tRNA_m^{Met} and other aminoacyl-tRNAs (Ono et al., 1968).

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